New method for detecting acid-resistant microorganisms in the stool

In the specification of this invention a number of published documents are stated. The subject matter of these documents is herewith incorporated into the specification by reference.

The invention relates to a method for detecting an infection of a mammal with an acid-resistant microorganism, wherein (a) a stool sample of a mammal is incubated with at least two different monoclonal antibodies, fragments or derivatives thereof or aptamers under conditions allowing a complex formation of antigens of the acid-resistant microorganism with antibodies, fragments or derivatives thereof or the aptamers, and wherein (aa) the first monoclonal antibody or the fragment or the derivative thereof or the first aptamer specifically binds an epitope of the first antigen, which shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; (ab) the second monoclonal antibody or the fragment or the derivative thereof or the second aptamer specifically binds an epitope of a second antigen differing from the epitope of the first antigen, which shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide, wherein the parts of the mammals may overlap according to (aa) and (ab) and in total essentially make up the overall number of infected mammals; and (b) the formation of at least one antigen-antibody complex/antigen-aptamer complex according to (aa) or (ab) is detected.

Preferably, the acid-resistant microorganism is a bacterium, especially *Helicobacter pylori, Helicobacter hepaticus or Mycobacterium tuberculosis* or *Campylobacter pylori*. Moreover, it is preferred that the two epitopes are epitopes of a urease and a heat shock protein, preferably of Hsp60, of an alkylhydroperoxide-reductase, preferably of the 26kDa-protein, the 20kDa-protein (3-dehydro-quinase, type II), of the 16.9kDa-protein (neutrophil-activating protein) or of the 33.8kDa-protein (fructose-bisphosphate aldolase). In another preferred embodiment of the invention, the stool sample is examined with three different monoclonal antibodies, fragments or derivatives thereof or aptamers, wherein the first antibody, the fragment or derivative thereof or the first aptamer is an epitope of a urease and the second and third antibodies (or the derivative, fragment or aptamer) each specifically bind an epitope of the different aforementioned proteins, preferably Hsp60 (if one of the two first epitopes is an epitope of an alkylhydroperoxide-reductase) or an alyklhydroperoxide-reductase, if one of the two first epitopes is an epitope of Hsp60. In addition, the invention relates to diagnostic and pharmaceutical compounds and test devices containing said components as well as the packaging containing them.

Today, there are different, invasive and non-invasive, direct and indirect ways to detect the infection of a mammalian organism with microbial pathogens or parasites. If an invasive technique is used, the physical integrity of the examined subject is violated, e.g. by biopsy or by taking a serum. Non-invasive diagnostic methods note changes in parameters which may be measured without interfering in the organism. Preferably, samples of body fluids and excretions such as breathing air, urine, saliva, sweat or stool are taken and analysed. With direct methods the presence of the pathogen or parasite, its components or its degradation products may be detected, e.g. by staining and microscopic examination or specific enzymatic reactions. Indirect methods are used for detecting reactions of the host organism and the pathogen or the parasite, e.g. the presence of antibodies against antigens of the pathogen in the serum, urine or saliva of the host. In another indirect method, the patient has to be supplied with indicator substances which the microbial pathogens or parasites modify in a specific way, with the modified form being detected in samples (breath test).

Interfering in the organism, for instance when taking a tissue sample, strains the organism in most of the cases and often requires a great number of instruments and involves risks to the health. Thus, non-invasive techniques are preferred

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since it is comparatively easy to take samples of the above-mentioned body fluids and excretions. Furthermore, as not every host reacts in the same way to a certain pathogen or parasite and as the host's reaction is delayed and may also persist after the pathogen of parasite has been removed from the organism, direct methods should always be preferred. Ideally, a diagnosis is made by means of the non-invasive, direct detection of the pathogen or parasite in body fluids or excretions.

Moreover, a diagnostic method should also be optimised as regards other points: high reproducibility, sensitivity and specificity, guaranteed availability and constant quality of the materials to be used, low costs for the production and for carrying out the method and simple application independent of complex instruments are parameters to be taken into consideration.

For the above-mentioned reasons, in medical diagnostics increasing use is made of methods based on the high selectivity and binding affinity of certain classes of substances (e.g. antibodies, receptors, lectines, aptamers) for molecular structures which may be selected in such a way that they are highly specific for the substance/microorganism that is to be determined. It was mainly the possibility of immobilising these substances at the surface of solids as well as the coupling with radioactive nuclides, with enzymes triggering colour reactions when combined with suitable substrates, or with coloured particles with a highly specific binding affinity (e.g. ELISA = enzyme-linked immunosorbent assay) that led to the development of cheaper, simpler and less timely methods for detecting substances peculiar and foreign to body.

In the initial phases of the development of these detection methods exclusively polyclonal antibodies were used. They, however, have several disadvantages well known to the person skilled in the art, chief among these being limited availability and often cross reactivity. These disadvantages could be eliminated to a very great extent by developing methods for producing monoclonal antibodies (Köhler & Milstein (1975)), making progress as regards the isolation of receptors and their directed expression in cellular host systems, developing lectines with a high affinity for certain carbohydrates as well as by discovering that nucleic acids (aptamers) can specifically bind molecular structures. Today, the specificity and sensitivity of detection methods may be optimised with comparatively simple methods.

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Due to the high specificity, such methods are particularly suitable for detecting single, defined substances like haptens, peptides or proteins, provided the structural element that has been recognised is constant within the population of the sample that is to be analysed and specific for the substance that is to be detected. Furthermore, they are suitable for measurements in body fluids and thus they are an obvious option for the direct detection of pathogens in this sample matrix. Accordingly, methods for the diagnosis of e.g. *Entamoeba histolytica* (Haque (1993), J. Infect. Dis. **167**: 247-9), enterohemorrhagic *Escherichia coli* (EHEC; Park (1996), J. Clin. Microbiol. **34**: 988-990), *Vibrio cholerae* (Hasan (1994), FEMS Microbiol. Lett. **120**: 143-148), particles similar to the Toro virus (Koopmans (1993), J. Clin. Microbiol. **31**: 2738-2744) or *Taenia saginata* (Machnicka (1996), Appl. Parasitol. **37**: 106-110) in the stool have been described in the state of the art. A diagnostic method for gastroenteritis caused by adenovirus (Herman (1987), J. Infect. Dis. **155**: 1167-1171) combines two monoclonal antibodies that are directed against different enteric serotypes of adenovirus in order to be able to detect these serotypes uninfluenced by the presence of other non-enteric adenoviruses. In this case, provided the serotype of the adenovirus with which the patient is indentified is known, a reliable detection with the respective monoclonal antibody alone is possible.

A common feature of said pathogens is that they are viable and reproducible in the intestine, the intestinal mucosa or in the gut lumen of the host, in all cases of human. Thus, they have mechanisms allowing them to survive and multiply in the presence of the degradation and digestion systems active in the intestine. Therefore, a great number of intact or almost intact pathogens or parasites are likely to be passed with the stool. It is easy to detect them in the stool or in prepared stool samples by means of detection reagents, e.g. antibodies that recognise components of the pathogens or parasites.

There is, however, a number of pathogens and parasites that, on the one hand, may be present in the stool due to their relations of the tissue infested (e.g. lung, stomach, pancreas, duodenum, liver) to the gastrointestinal tract and that on the other hand are not viable and/or reproducible in the intestine itself. Here, these pathogens and parasites are called acid-resistant microorganisms. Among these pathogens and parasites are, for instance, *Helicobacter pylori (H. pylori)* and *Helicobacter hepaticus, Mycobacterium tuberculosis* and other mycobacteria,

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Chlamydia pneumoniae, Legionella pneumophilae, Pneumocystis carinii, Campylobacter jejuni, Campylobacter pylori and others. Some of these pathogens may be detected, for example, in the sputum. It is, however, possible to detect for example *Mycobacterium tuberculosis* in the sputum only within a short period of time, i.e. after a cavern containing the pathogen has opened. Moreover, detection is rendered more difficult due to the fact that it is not always possible to get a sputum sample of the subject to be examined. This applies to infants, confused patients or animals. Other pathogens like *Legionella pneumophilae* can be detected specifically by means of antigens which get into the urine via the kidney. Yet, this is only possible if the amount contained in the urine is sufficient for the detection. Detection in the stool would be a good alternative. In these organisms, however, the intestinal passage is combined with a strong attack by the mammal's digestion and degradation mechanisms, especially the intestinal flora. Thus, molecular structures that are specific to the pathogen observed may be destroyed or their concentration may be strongly reduced.

With acid-resistant bacteria, the degradation of pathogens in the intestine has turned out to be a problem for reliable detection in stool samples. The number of germs that get into the stomach of the infected patient is low compared to the number of other bacteria colonising the intestine. Furthermore, germs and germ fragments have to pass a long way through the intestine that is rich in proteases after leaving the stomach. Due to these circumstances, only small amounts of intact proteins are found in the stool. This also causes the fact that the combination of two epitopes on one antigen, which is necessary for an ELISA, is no longer necessarily like the one occurring in the native protein, and that epitopes located closely together are most likely to show a positive result in a detection method requiring two epitopes on the same molecule. In addition, the distribution of antigens detected in the stool of patients infected, which is individually different, indicates individual features in the processing of the antigens in the intestinal passage. The first approach to reduce this problem was offered by the disclosure of EP-A 0 806 667. In this application it is shown that polyclonal antibodies could be induced with the lysate of a certain *H. pylori* strain. These antibodies recognise a greater variability of strains from different geographic regions. The application ,however, does not show which antigens are recognised by the serum. Furthermore, the corresponding diagnosis by means of monoclonal antibodies was excluded. In view of the fact that the immunosera may vary in spite of all standardisation efforts, the method developed in the above-mentioned

application must be regarded as suboptimal for broad application. In addition, it is necessary to keep immunising new animals in order to provide polyclonal sera. The corresponding methods require a great amount of time and cost.

Thus, the technical problem underlying the present invention was to improve the aforementioned disadvantageous methods of the prior art and to provide a method for reliably detecting acid-resistant microorganisms, which is economical and easy to standardise.

This technical problem has been solved by providing the embodiments characterised in the claims.

Therefore, the invention relates to a method for detecting an infection of a mammal with acid-resistant microorganisms, wherein

(a)   a stool sample of a mammal is incubated with at least two different monoclonal antibodies, fragments or derivatives thereof or aptamers under conditions allowing a complex formation of antigens of the acid-resistant microorganism with antibodies, fragments or derivatives thereof or the aptamers, and wherein

   (aa)   the first monoclonal antibody or the fragment or the derivative thereof or the first aptamer specifically binds an epitope of the first antigen, which shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide;

   (ab)   the second monoclonal antibody or the fragment or the derivative thereof or the second aptamer specifically binds an epitope of a second antigen differing from the epitope of the first antigen, which shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a

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synthetic peptide, wherein the parts of the mammals may overlap according to (aa) and (ab) and in total essentially make up the overall number of infected mammals; and

(b)   the formation of at least one antigen-antibody complex or antigen-aptamer complex according to (aa) or (ab) is detected.

According to the invention, the term "acid-resistant microorganism" contains any microorganism that, due to its properties/mechanisms of adapting to the host, withstands the physical and chemical influence of the digestive tract in such a way that it is detectable by a preferably immunological test or by the use of aptamers. Examples of such acid-resistant microorganisms are *Helicobacter hepaticum, Mycobacterium tuberculosis, Mycobacterium pseudotuberculosis, Mycobacterium cansassii, Campylobacter jejuni* and *Campylobacter pylori.*

The term "stool sample of a mammal" means according to the invention any stool sample that may be used for the detection method of the invention. It preferably includes stool samples that have been prepared for diagnostic tests according to methods essentially known. Preparation may be carried out for example according to RIDASCREEN® Entamoeba enzyme immunoassay (R-Biopharm GmbH, Darmstadt).

According to the invention, the terms "fragments" or "derivatives" of monoclonal antibodies have the same binding specificity as monoclonal antibodies. Such fragments or derivatives may be produced according to common techniques; e.g. Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, USA, 1988. Examples of fragments are Fab-, $F(ab')_2$ or Fv-fragments. Examples of derivatives are scFv-fragments. Derivatives may also be substances which have been produced chemically and which have the same or improved binding properties as the antibodies. Such substances may be produced for instance by peptidomimetics or various rounds of phage display and subsequent selection as to improved binding properties. Aptamers are according to the invention nucleic acids such as RNA, ssDNA (ss = single strand), modified RNA or modified ssDNA, which bind a great variety of target sequences with high specificity and affinity. The term "aptamer" has been known and described in the prior art, e.g. in Osborne et al., Curr. Opin. Chem. Biol. 1 (1997), 5-9 or in Stull and Szoka, Pharm. Res. 12 (1995), 465-483.

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There is no upper limit for the term "at least two" as regards the number of antibodies used, i.e. for example three, four, five, six, seven, eight, nine or ten antibodies etc.

The person skilled in the art may adjust "conditions allowing a complex formation" without much ado; cf. Harlow and Lane, ibid. These conditions may, for instance, be physiological conditions.

According to the invention the term "shows [...] a structure after the intestinal passage that corresponds to the native structure" means that the epitope of an antigens is recognised after the intestinal passage by a monoclonal antibody, derivative or fragment thereof or the aptamer, which has been obtained against or which is bound to the same antigen/epitope that has not passed the intestinal passage. In other words, the epitope/antigen that is specifically bound by said antibody or fragment, or derivative thereof or aptamer has passed the intestinal passage intact or almost intact as regards its structure and has not been degraded. A source for the native structure of the epitope/antigen may, for instance, be a bacterial extract that was disrupted with a French press and further purified according to standard methods (cf. Sambrook et al. "Molecular Cloning, A Laboratory Manual", 2nd edition 1989, CSH Press, Cold Spring Harbor, USA), or a lysate which has been further purified according to standard methods (e.g. Sambrook et al., ibid).

The term "shows [...] a structure after the intestinal passage that corresponds to [...] the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide" means according to the invention that the epitope recognised by the monoclonal antibody, fragment, derivative or aptamer corresponds to an epitope that is presented by the immune system of a mammal, preferably of a human. The mechanisms of antigen presentation as well as the mechanisms leading to the processing of antigens and the variety of antibodies resulting therefrom has been known to the prior art and has been described, for instance, in Janeway and Travers, Immunologie, 2nd edition 1997, Spektrum Akademischer Verlag GmbH, Heidelberg. These epitopes may differ from native epitopes. The mammal's contact with the microorganisms or the proteins or fragments or the synthetic

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peptides may be in the form of an infection (except for synthetic peptides) or in the form of immunisation. For immunisation also extracts, lysates, synthetic peptides, etc. of the microorganism/protein may be used. Appropriate immunisation methods have been known to the prior art and have been described, for example, in Harlow and Lane, ibid. Suitable antibodies may also be obtained for example by immunisation and/or screening on surrogates like synthetic peptides, recombinantly produced proteins, extracts, lysates or partially digested proteins.

According to the invention, the term " specifically binds" means that the monoclonal antibody, the fragment or derivative thereof or aptamer shows no or essentially no reactivity with other epitopes in samples of non-infected mammals.

The term " wherein the groups of mammals may overlap according to (aa) and (ab) and in total essentially make up the overall number of infected mammals" means that at least one, possibly also two or more epitopes/antigens may be found in the stool, which are specifically bound by the monoclonal antibodies, fragments or derivatives or the aptamers and that the monoclonal antibodies, fragments or derivatives or the aptamers detect at least one antigen in essentially every infected mammal. In this case, the term "in total essentially [...] the overall number" means that the epitopes and thus a corresponding infection with the microorganism may be recorded with more than 70 %, preferably at least 75 %, more preferably more than 85 %, particularly preferred more than 90 % and most preferably more than 95 % of the subjects affected. Ideally, infections are detected with 100 % of the subjects affected.

According to the present invention, the term "antigen-antibody complex" does not only comprise complexes the antigen forms with the native antibody, but also complexes it forms with the fragments or derivatives of the antigen.

Surprisingly, it was found according to the invention that by means of a limited number of monoclonal antibodies, fragments or derivatives thereof or aptamers, which specifically bind at least two different epitopes of different antigens of acid-resistant microorganisms, an infection with these bacteria/pathogens may be diagnosed in a relatively reliable way. In this embodiment of the invention, antigens of a prepared stool sample, for instance, may be linked to a solid phase and the infected agent may be detected with the limited number of monoclonal antibodies, fragments or derivatives thereof or aptamers, which have been

labelled. If the antigen in the intestinal passage is (still) present in a dimeric or multimeric form, the same monoclonal antibodies, fragments or derivatives thereof or aptamers can be used both as catchers and detectors. This embodiment also comprises the possibility of one epitope being present on the same subunit several times. The invention includes embodiments in which further epitopes having the aforementioned properties are recognised by further monoclonal antibodies or fragments or derivatives or by further aptamers. In addition, an antigen may be detected by several monoclonal antibodies or aptamers which recognise the same or different epitopes of the antigen. Three monoclonal antibodies or three aptamers, for instance, may detect three different epitopes on two protein fragments. The latter embodiments are suitable for further increasing the reliability of the diagnosis. In the case of structures of a higher category, e.g. of dimers or multimers, the same monoclonal antibody can, in the method of the invention, also bind several epitopes in the same multimeric protein. Examples of such embodiments are subsequently described in more detail.

The invention also comprises embodiments in which only monoclonal antibodies or fragments or derivatives thereof or only aptamers are used, as well as embodiments in which different kinds of detection reagents are used in a test. Thus, it is possible to use the first monoclonal antibody with the second antibody derivative or the first aptamer and the second antibody fragment, to state only two examples. To that extent, the terms "first" and "second" mean the first and second detection reagents. This, however, does not mean that always two antibodies, derivatives or fragments thereof or two aptamers are used.

The results of the invention are surprising mainly because the state of the art has taught away therefrom. In the case of *H. pylori*, for example, it was found that main antigens do not show the desired specificity and sensitivity in ELISA; cf. Newell et al., Serodiag. Immunother. Infect. Dis. **3** (1989), 1-6. Furthermore, EP-A 0 806 667 teaches that it is not possible to detect *H. pylori* infections with monoclonal antibodies due to the genetic variability of the *H. pylori* strains.

The method of the invention is of advantage compared to the aforementioned state of the art mainly since it is possible to obtain a relatively reliable diagnosis with only two monoclonal antibodies or fragments or derivatives thereof or aptamers. Preferably, pairs of antibodies, fragments, derivatives thereof or aptamers are used for detection, for example in ELISA, wherein the two

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antibodies of the pair bind the same or different epitopes on the same antigen. *H. pylori* urease, for instance, forms multimeric structures of several identical as well as non-identical subunits. Therefore, in ELISA or other assays, the same antibodies, fragments or derivatives thereof or aptamers can be used both as catching-antibodies/-aptamers and as detection-antibodies/-aptamers. On the one hand, using monoclonal antibodies, fragments or derivatives thereof or aptamers ensures a standard as regards the reliability of the diagnostic method that is easy to maintain, which is a great advantage compared to diagnostic methods that have been known so far or that have been introduced for this purpose. In addition, it is no longer necessary to keep immunising and subsequently testing new test animals as is required, for example, in EP-A 0 806 667. Another advantage of the method according to the invention is the fact that it can be carried out as a direct and non-invasive method, which enhances the aforementioned positive effects for the patients and increases the reliability when it comes to determining the stage of the disease.

In a preferred embodiment, the acid-resistant microorganism is an acid-resistant bacterium.

A number of acid-resistant bacteria have been known in the state of the art. In a particularly preferred embodiment, the bacterium belongs to the genus Helicobacter or the genus Mycobacterium or the genus Campylobacter.

In another particularly preferred embodiment, the bacterium is a bacterium belonging to the species *Helicobacter pylori, Helicobacter hepaticum* or a bacterium belonging to the species *Mycobacterium tuberculosis* or a bacterium belonging to the species *Campylobacter jejuni* or *Campylobacter pylori*.

In another preferred embodiment, the epitope of the first antigen is an epitope of an urease, e.g. an α-urease or a β-urease and the epitope of the second antigen is an epitope of a heat shock protein, an alkylhydroperoxide-reductase or of the 20kDa-protein (3-dehydro-quinase, type II), of the 16.9kDa-protein (neutrophil-activating protein) or of the 33.8kDa-protein (fructose-bisphosphate-aldolase).

According to the invention, it was surprisingly found that in a population of mammals, especially of human patients, the stool of which was tested for infections with acid-resistant bacteria, essentially all members of this population

had at least one of said epitopes in the stool, so that a relatively reliable diagnosis can be made using a set of only two corresponding monoclonal antibodies, fragments of derivatives thereof or aptamers.

The urease is particularly preferred to be the β-urease of *H. pylori*.

In another particularly preferred embodiment, the heat shock protein is a Hsp60.

Furthermore, the Hsp60 is particularly preferred to be the Hsp60 of *H. pylori*.

In another particularly preferred embodiment, the alkylhydroperoxide-reductase is the 26kDa-protein of *H. pylori*.

The other said proteins are also preferred to be proteins of *H. pylori*.

In another (preferred) embodiment, the method of the invention for detecting an infection of a mammal with an acid-resistant microorganism comprises the following steps, wherein (a) a stool sample of a mammal is incubated with three different monoclonal antibodies, fragments or derivatives thereof or aptamers under conditions allowing the complex formation of antigens from the acid-resistant microorganism with antibodies, fragments or derivatives thereof or the aptamers and wherein (aa) the first monoclonal antibody or the fragment or derivative thereof or the first aptamer specifically binds an epitope of the first antigen, which shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; (ab) the second monoclonal antibody or the fragment or derivative thereof or the second aptamer specifically binds an epitope of a second antigen differing from the epitope of the first antigen, which shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide; and (ac) the third monoclonal antibody or the fragment or derivative thereof or the third aptamer specifically binds an epitope of a third antigen differing from the epitope of the first and second antigen, which

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shows at least with some mammals a structure after the intestinal passage that corresponds to the native structure or the structure which a mammal produces antibodies against after being infected or immunised with the acid-resistant microorganism or an extract or lysate thereof or a protein therefrom or a fragment thereof or a synthetic peptide, wherein the groups of mammals may overlap according to (aa), (ab) and (ac) and in total essentially make up the overall number of infected mammals; and the formation of at least one antigen-antibody complex/antigen-aptamer complex according to (aa), (ab) or (ac) is detected.

As regards the definition and the preferred embodiments of this method we point to the description of the aforementioned embodiments. The explanations as to the combination of various detection reagents when using at least two different reagents correspondingly apply for three detection reagents. Thus, in a test, the first monoclonal antibody may be combined with the second fragment and the third aptamer. This combination, however, does not mean that there are another two aptamers used in the test. Therefore, the term "third" refers to the third detection reagent, independently of its possible structure. The same applies to the terms "first" and "second".

Preferably, the epitope of the first antigen is an epitope of a urease, preferably of the β-urease of *H. pylori*, the epitope of the second antigen is an epitope of a heat shock protein, preferably of Hsp60, preferably of *H. pylori* and the epitope of the third antigen is an epitope of an alkylhydoperoxide- reductase, preferably of the 26kDa-protein of *H. pylori*. In other preferred embodiments, the epitopes of the second and the third antigen are epitopes of the 20kDa-protein (3-dehydro-quinase, type II), the 16.9kDa-protein (neutrophil-activating protein) or of the 33.8kDa-protein (fructose-bisphosphate-aldolase), in combination with the urease as a first and optionally the Hsp60 or the 26kDa-protein as a second or third antigen. In another preferred embodiment, the epitopes of the second antigen are epitopes of Hsp60 and the epitopes of the third antigen are epitopes of the 20kDa-protein (3-dehydro-quinase, type II), the 16.9kDa-protein (neutrophil-activating protein) or the 33.8kDa-protein (fructose-bisphosphate-adolase). Other embodiments are possible, even without urease, by detecting combinations of said proteins or their epitopes.

In serial analyses of stool samples of patients infected with *H. pylori* for various *H. pylori*-specific proteins (urease, Hsp60, alkylhydroperoxide-reductase) by

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means of ELISA using monoclonal antibodies it was found that the detection of different antigens in the stool samples of various patients was very inhomogeneous. In addition, it surprisingly was found that the combination of monoclonal antibodies that recognise partly overlapping epitopes were very suitable for the detection of these antigens by means of Sandwich-ELISA (see Table 3).

According to the invention, the monoclonal antibodies, fragments or derivatives thereof or the aptamers can recognise and specifically bind linear or conformation epitopes. In another preferred embodiment, at least one of the monoclonal antibodies or one of the fragments, derivatives or aptamers binds a conformation epitope.

In a particularly preferred embodiment, all monoclonal antibodies, etc. bind conformation epitopes.

Five of the murine mAK (monoclonal antibodies) that were particularly suitable for the stool-ELISA were analysed with epitope-mapping (Table 3). Surprisingly, for three of these antibodies it was not possible to determine a linear epitope. Thus, it was assumed that these mAK recognise conformation epitopes. As regards the anti-β-urease, this statement is confirmed by the results of the immunoblotting which show that the denatured urease is recognised no longer or only to a very limited extent (see Table 2).

In another embodiment, the invention relates to a method for detecting an infection with *Helicobacter pylori* in the stool of a mammal, wherein (a) a stool sample of a mammal is incubated with at least two different monoclonal antibodies, fragments or derivatives thereof or aptamers under conditions allowing the formation of an antigen-antibody/antigen-aptamer complex wherein (aa) the first monoclonal antibody, the fragment or derivative thereof or the first aptamer specifically binds β-urease or a fragment thereof; (ab) the second monoclonal antibody, the fragment or derivative thereof or the second aptamer specifically binds the 26kDa-antigen or a fragment thereof or specifically binds Hsp60 or a fragment thereof; and (b) the formation of at least one antigen-antibody complex/antigen-aptamer complex according to (aa) or (ab) is detected.

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Furthermore, the invention relates to a method for detecting an infection with *Helicobacter pylori* in the stool of a mammal, wherein (a) the stool sample is incubated with three different monoclonal antibodies, fragments, derivatives thereof or aptamers under conditions allowing the formation of an antigen-antibody/antigen-aptamer complex, wherein (aa) the first monoclonal antibody, the fragment, derivative thereof or the first aptamer specifically binds β-urease or a fragment thereof; (ab) the second monoclonal antibody, the fragment, derivative thereof or the second aptamer specifically binds Hsp60 of a fragment thereof; and (ac) the third monoclonal antibody, the fragment, derivative thereof or the third aptamer specifically binds the 26kDa-antigen or a fragment thereof; and (b) the formation of at least one antigen-antibody/antigen-aptamer-complex is detected according to (aa), (ab) or (ac).

As regards the definitions used and the preferred embodiments that may be used, we refer to the aforementioned statements.

The proteins stated in the two aforementioned embodiments are of course *H. pylori*-proteins.

In another preferred embodiment, the Hsp60-specific antibody is the antibody which has been generated by the hybridoma HP16m/2A5-E6-E5 filed with the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ) on June 23, 1998 in accordance with the Statutes of the Budapest Treaty under the filing number DSM ACC2356.

In another preferred embodiment, the 26kDa-antigen-specific antibody is the antibody generated by the hybridoma HP15m/3E8-D9-D6 filed with the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ) on June 23, 1998 in accordance with the Statutes of the Budapest Treaty under the filing number DSM ACC2355.

In another preferred embodiment, the β-urease-specific antibody is the antibody generated by the hybridomas HP8m/4H5-D4-C9 or HP9.1m/3C2-F8-E2 filed with the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ) on June 23, 1998 in accordance with the Statutes of the Budapest Treaty under the filing numbers DSM ACC2360 or DSM ACC2362. The β-urease-specific antibody HP8m/1H5-G2-B4 is generated

by a daughter clone of the filed hybridoma HP8m/4H5-D4-C9. The two antibodies produced by parent and daughter clones are encoded by identical DNA sequences and have the same properties.

In another particularly preferred embodiment, the heavy chain of the antibody binding an Hsp60-epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

    CDR1:   GFSLSRYSVH
    CDR2:   MIWGGGSTDYNSGLKS
    CDR3:   NMGGRYPDYFDY

In another particularly preferred embodiment, the DNA sequence encoding the heavy chain of the antibody binding an Hsp60-epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the following CDRs:

    CDR1:   GG GTTCTCATTA TCCAGATATA GTGTACAC
    CDR2:   ATGATATGGG GTGGTGGAAG CACAGACTAT AATTCAGGTC
            TCAAATCC
    CDR3:   AATATG GGGGGTAGGT ACCCGGACTA CTTTGACTAC

In another preferred embodiment, the light chain of the antibody binding an Hsp60-Epitope has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

    CDR1:   RASKSVSTSGYSYIH
    CDR2:   LASNLES
    CDR3:   QHSRELPLT

Furthermore, in another particularly preferred embodiment, the DNA sequence encoding the light chain of this antibody has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

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CDR1: A GGGCCAGCAA GAGTGTCAGT ACATCTGGCT ATAGTTACAT ACAC
CDR2: C TTGCATCCAA CCTAGAATCT
CDR3: CAGC ACAGTAGGGA GCTTCCGCTC ACG.

In another particularly preferred embodiment of the method of the invention, the heavy chain of the antibody binding a 26kDa-protein has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

CDR1: GFTFNSYAMY
CDR2: RIRSKSDNYATYYANSVKD
CDR3: DHDKFPFYYALDY

In another particularly preferred embodiment, the DNA sequence encoding the heavy chain of the antibody binding the epitope of the 26kDa-protein has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

CDR1: GG TTTCACCTTC AATTCCTATG CCATGTAC
CDR2: CGCATAAGAA GTAAAAGTGA TAATTATGCA ACATATTATG CCAATTCAGT GAAAGAC
CDR3: GATCATG ATAAGTTTCC TTTTTACTAT GCTCTGGACT AC

In another particularly preferred embodiment of the method of the invention, an antibody, fragment or derivative thereof is used the light chain of the antibody binding the 26kDa-protein has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

CDR1: TASSSVSSSYLH
CDR2: STSNLAS
CDR3: HQYHRSPPT

In addition, the DNA sequence encoding the light chain of the antibody has in another particularly preferred embodiment at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

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CDR1:   A CTGCCAGCTC AAGTGTGAGT TCCAGTTACT TGCAC
CDR2:   AGCACTTCCA ACCTGGCTTC T
CDR3:   CAC CAGTATCATC GTTCCCCACC GACG

In another particularly preferred embodiment of the method of the invention, the heavy chain of the antibody binding an epitope of the β-urease has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

CDR1:   GFTFSSHFMS
CDR2:   SISSGGDSFYPDSLKG
CDR3:   DYSWYALDY
or:
CDR1:   GYAFSTSWMN
CDR2:   RIYPGDGDTNYNGKFKG
CDR3:   EDAYYSNPYSLDY

In another particularly preferred embodiment the DNA sequence encoding the heavy chain of the antibody binding an epitope of the β-urease has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

CDR1:   GG CTACGCATTC AGTACCTCCT GGATGAAC
CDR2:   CGGATTTATC CTGGAGATGG AGATACTAAC TACAATGGGA AGTTCAAGGG C
CDR3:   GAG GATGCCTATT ATAGTAACCC CTATAGTTTG GACTAC
or:
CDR1:   GG ATTCACTTTC AGTAGCCATT TCATGTCT
CDR2:   TCCATTAGTA GTGGTGGTGA CAGTTTCTAT CCAGACAGTC TGAAGGGC
CDR3:   GACTAC TCTTGGTATG CTTTGGACTA C

In another particularly preferred embodiment of the method of the invention, the light chain of the antibody binding an epitope of the β-urease has at least one of the following CDRs, preferably the CDR3 and still more preferably all of the three following CDRs:

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CDR1:    RASQSIGTRIH
CDR2:    YGSESIS
CDR3:    QQSNTWPLT
or:
CDR1:    HASQNINVWLS
CDR2:    KASNLHT
CDR3:    QQGRSYPLT
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Moreover, the DNA sequence encoding the light chain of this antibody preferably has the following CDRs:

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CDR1:    A GGGCCAGTCA GAGCATTGGC ACAAGAATAC AC
CDR2:    TAT GGTTCTGAGT CTATCTCT
CDR3:    CAACAA AGTAATACCT GGCCGCTCAC G
or:
CDR1:    C ATGCCAGTCA GAACATTAAT GTTTGGTTAA GC
CDR2:    AAG GCTTCCAACT TGCACACA
CDR3:    CAACAG GGTCGAAGTT ATCCTCTCAC G
```

Furthermore, it is particularly preferred that the heavy and light chains showing said CDRs occur together in one antibody, fragment or derivative thereof, which specifically binds the Hsp60, the 26kDa-protein or the β-urease or a fragment thereof, preferably of *H. pylori*. The invention, however, also comprises embodiments in which these heavy and light chains are combined with other light or heavy chains, wherein the binding properties may generally be preserved or enhanced. Corresponding methods have been known in the prior art. Particularly preferred antibodies have in the variable regions of the light and heavy chains the amino acid sequences shown in Figures 1 and 2, Figures 3 and 4, Figures 5 and 6 or Figures 7 and 8, or the regions are encoded by the DNA sequences depicted.

In a particularly preferred embodiment, the following steps are carried out with the stool sample before incubation with the antibodies: the stool sample is resuspended with a resuspension buffer in a ratio of 1:3 to 1:25, preferably of 1:10, then it is mixed on a vortex mixer. An example of a resuspension buffer is: 150 mM PBS, 0.1 % SDS.

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In another preferred embodiment, the formation of the at least one antigen-antibody complex/antigen-aptamer complex in step (b) is detected by means of an immunological method.

In another preferred embodiment, the formation of the at least one antigen-antibody complex/antigen-aptamer complex in step (b) is detected by means of ELISA, RIA, Western Blot or an immunochromatographic method.

Such methods have been known in the prior art; cf. Harlow and Lane, ibid.

In another particularly preferred embodiment of the method of the invention, in the immunological method, especially in RIA or ELISA, the same antibody or the fragment or derivative thereof or the aptamer is used both for binding to the solid phase and for detecting the epitope. While the catching antibody/catching aptamer may be bound to the solid phase, e.g. a micro titre-plate, in an unmodified form, the antibody, the fragment or derivative thereof or the aptamer, which used for the detection, is optionally labelled. On the other hand, this antibody, the fragment or derivative thereof or this aptamer may also not be labelled and thus the epitope of the microorganism, preferably the bacterial epitope, may also be detected through a third labelled antibody, the fragment or derivative thereof or a third labelled aptamer , wherein this antibody, the fragment or derivative thereof or this aptamer may be a species-specific or Ig-class-specific antibody or a corresponding aptamer. Labellings, e.g. with radioactive or fluorescent labels, have been known in the prior art; cf. Harlow and Lane, ibid. The same applies to aptamers. The above-described embodiment is particularly suitable for detecting the urease, preferably β-urease, which optionally may also be found after the intestinal passage as a dimer, optionally in a multimeric form. In this embodiment, of course, also combinations of antibodies, fragments, derivatives and aptamers may be used, e.g. combinations of antibodies, etc. that bind to different epitopes of the same antigen.

In another preferred embodiment of the method of the invention, the monoclonal antibody is a murine antibody.

In addition, in another preferred embodiment, the antibodies, fragments or derivatives thereof or the aptamers are fixed to a support.

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When carrying out routine checks, it is of particular advantage to fix the antibodies, fragments or derivatives thereof or the aptamers to a support. Moreover, the combination antibody-support/aptamer-support may be packaged as a kit or in the form of a kit.

In another particularly preferred embodiment, the material of the support is a porous material.

In another particularly preferred embodiment the support material is a test strip.

In addition, in a preferred embodiment, the support consists of cellulose or a derivative of cellulose.

The mammal the stool of which may be analysed with the method of the invention may be an animal, e.g. a domestic animal such as a cat or a dog, a useful animal such as a pig or other kinds of animals such as a mouse, a tiger or a ferret.

In another preferred embodiment, the mammal is a human.

Furthermore, the invention relates to a monoclonal antibody, a fragment or derivative thereof that has a V region which has a combination of said CDRs or which was produced by one of said hybridomas.

In this case, a monoclonal antibody, fragment or derivative thereof is preferred which has at least one of the V regions depicted in Figures 1 to 8. Preferably, this antibody has two of the V regions shown in Figures 1 and 2, 3 and 4, 5 and 6 or Figures 7 and 8. Moreover, these V regions are preferred to be encoded by the DNA sequences shown in Figures 1 to 8.

In a particularly preferred embodiment of the invention, the monoclonal antibody, the fragment or derivative thereof is a murine antibody or a fragment or derivative thereof or a chimeric, preferrably a humanised antibody or a fragment or a derivative thereof. The derivative may also be a fusion protein. Furthermore, the antibody is preferred to be labelled, for instance with a colloid, with a radioactive, fluorescent, phosphorescent or chemiluminescent labelling.

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The generation of chimeric humanised and human antibodies and of the other derivatives has been known well in the state of the art (e.g. Vaughan et al., 1998; Orlandi et al., 1989; Harlow and Lane, ibid).

The invention also relates to an aptamer which specifically binds the same epitope as the monoclonal antibody, the fragment or derivative thereof. Such aptamers may be generated according to methods known well in the state of the art.

In addition, the invention relates to an epitope that is specifically bound by one of said monoclonal antibodies, fragments or derivatives thereof or an aptamer.

Furthermore, the invention relates to other antibodies, fragments or derivatives thereof, which specifically bind the epitope of the invention. These antibodies may, for example, be monoclonal antibodies which have been generated accordigng to standard methods using the epitope as hapten/component of an antigen.

Moreover, the present invention relates to a diagnostic composition containing at least two monoclonal antibodies, fragments or derivatives thereof or aptamers as have been defined before, optionally fixed to a support.

Furthermore, the present invention relates to a test device for detecting at least one of said epitopes, comprising (a) at least two monoclonal antibodies, fragments or derivatives thereof or aptamers as defined above, fixed to a support; (b) a device for preparing and analysing stool samples and optionally (c) a mixture of at least two monoclonal antibodies, fragments or derivatives thereof or aptamers.

A further subject matter of the invention is a test device comprising (a) at least two monoclonal antibodies, fragments or derivatives thereof or aptamers as described above, wherein the antibodies, fragments or derivatives thereof or aptamers are conjugated with colloidal gold, latex particles or other colouring particles the size of which is typically between 5 nm and 100 nm, preferably between 20 nm and 60 nm; (b) a device for preparing and analysing stool samples and optionally (c) a mixture of at least two monoclonal antibodies, fragments or derivatives thereof or aptamers.

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In addition, the invention relates to a kit comprising (a) at least two monoclonal antibodies, fragments or derivatives thereof or aptamers as defined above, optionally fixed to a support; optionally (b) a device for preparing and analysing stool samples and optionally (c) a mixture of at least two monoclonal antibodies, fragments or derivatives thereof or aptamers.

The invention also relates to a composition comprising at least one of said antibodies or one of said fragments, derivatives or aptamers, optionally in combination with a support that is pharmaceutically tolerable and/or a diluent. The composition is preferred to be a drug.

The person skilled in the art knows examples of appropriate pharmaceutically tolerable supports. These comprise phosphate-buffered saline solutions, water, emulsions such as oil/water-emulsions, different kinds of detergents, sterile solutions, etc. Drugs comprising such supports may be formulated by means of known, conventional methods. These drugs may be administered to an individual in an appropriate dose reaching, for example, from 1 µg to 100 mg per day and patient. There may be various ways of administration, e.g. intravenous, intraperitoneal, subcutaneous, intramuscular, local or intradermal. The physician in charge will choose the dosage according to clinical factors. The person skilled in the art knows that the dosage depends on various factors such as size, body surface, age, sex or general state of the patient, but it also depends on the specific drug that is applied, the duration and the kind of application and on other drugs which possibly are applied at the same time.

Finally, the invention relates to a package comprising the diagnostic compound, the test device of the invention or the kit of the invention.

The components of the diagnostic compound , the test device of the invention and/or the kit of the invention may be packed in containers like vials or tubules, optionally in buffers and/or solutions. Possibly, one or several components may be packed in one container.

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The Figures illustrate:

Fig. 1: the DNA sequence encoding for a light chain of a Hsp60-specific monoclonal antibody (DMS ACC2356). In this case as well as otherwise in the application, the DMSZ-filing number refers to the hybridoma generating the monoclonal antibody. The amino acid sequence encoding is shown as well.

Fig. 2: the DNA sequence encoding for a heavy chain of a monoclonal antibody (DMS ACC2356) that is specific to Hsp60. The amino acid sequence encoding is shown as well.

Fig. 3: the DNA sequence encoding for a light chain of a monoclonal antibody (DMS ACC2355) that is specific to the 26kDa-protein. The amino acid sequence encoding is shown as well.

Fig. 4: the DNA sequence encoding for a heavy chain of a monoclonal antibody (DMS ACC2355) that is specific to the 26kDa-protein. The amino acid sequence encoding is shown as well.

Fig. 5: the DNA sequence encoding for a light chain of the first monoclonal antibody (DMS ACC2360) that is specific to urease. The amino acid sequence encoding is shown as well.

Fig. 6: the DNA sequence encoding for a heavy chain of the first monoclonal antibody (DMS ACC2360) that is specific to urease. The amino acid sequence encoding is shown as well.

Fig. 7: the DNA sequence encoding for a light chain of the second monoclonal antibody (DMS ACC2362) that is specific to urease. The amino acid sequence encoding is shown as well.

Fig. 8: the DNA sequence encoding for a heavy chain of the second monoclonal antibody (DMS ACC2362) that is specific to urease. The amino acid sequence encoding is shown as well.

The Examples explain the invention.

Example 1: Isolation of *H. pylori* antigens

Cultivation of *H. pylori*

H. pylori (strain NCTC 11637) were plated in petri dishes on Wilkins chalkers agar adding 10 % horse blood and Amphotericin B, Vancomycin and Cefsoludin (Sigma Chemicals) and incubated in an microaerophile atmosphere (Anaerocult GasPAk, Merck) at 37°C for 3 or 4 days. The content of 2 dishes was suspended in a 1 l-bottle (Schott) in 350 ml of BHIB medium adding the antibiotics as above, the medium was fumigated for 10 min with a gas mixture of 10 % CO_2, 5 % O_2, 85 % N_2 and the bottle was sealed . The culture was shaken in a rotary shaker for 2 days at 37°C, after 24 hrs the bottle was opened. Then, the content of the bottle was put aseptically in a 10 ml-bottle, filled up with 4,7 l BHIB-medium, the medium was fumigated with a gas mixture of 10 % CO_2, 5 % O_2, 85 % N_2, then the bottle was sealed. It was again shaken in a rotary shaker for 2 days at 37°C, after 24 hrs the bottle was opened. Subsequently, the whole volume is centrifuged at 11,000 g for 10 min, the supernatant is decanted and the bacteria pellet is weighed. In order to store the pellet, it was resuspended in a physiological saline solution adding 15 % glycerine in a ratio of 2:1 (weight:volume) and frozen at –80°C. In order to check the identity of the cultivated bacteria, a visual inspection of the bacteria as well as tests for urease, oxidase and catalase activity were carried out. It was proven that it is free of contamination by cultivating some suspension, taken before freezing, in air at 37°C for 48 hrs.

Example 2: General procedure for selecting suitable antigens for the detection of microorganisms

After cultivation a lysate of the microorganism is produced. The lysate is separated by means of gel filtration. Then, the proteins are isolated a large quantity of which is present in the lysate. By means of protein sequencing and comparing the sequences with appropriate databases, these proteins are identified. In this way, it is possible to determine characteristic and specific

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proteins for the respective microorganism. Then a suitable purification method is developed for these selected antigens.

Example 3: Preparation of *H. pylori* urease, alkylhydroperoxide-reductase and Hsp60

The preparation of the three *H. pylori* antigens urease, alkylhydroperoxide-reductase and Hsp60 corresponds to a modification of a known method (Eschweiler et al., 1993). 10 % butanol was added to frozen bacteria pellet at a ratio of 1:2 (weight:volume), put in a mixer, turned upside down and shaken for about 15 min until it was completely thawed. After sedimentation for 20 min at 20,000 g and 4°C, the supernatant was decanted and the pellet was again extracted as above with 10 % butanol for 30 min. After repeated sedimentation, as described above, the supernatants were purified and the pellet was frozen for further usage.

Isolation of urease and alkylhydroperoxide-reductase (as oligomers)

The combined clear supernatants were filtrated through a 0.8 µm-filter and immediately applied on a Source Q 16/10-column (with 20 mM HEPES, pH 7.0, equilibrated). Fractions that are eluted with 20 mM HEPES, pH 7.0, 2 M NaCl were promptly tested for urease activity by means of a quick assay (4 ml 1 M urea, 1 ml 0.2 M HEPES, pH 7.0 and 80 µl of a solution of 5 g/l phenol red were mixed and filled up with aqua dest. to 20 ml. Then, 100 µl of this solution were filled with the same amount of a fraction in a test tube. The change in colour from red to yellow showed that urease was present). Urease-positive fractions were applied on a chromatography column filled with Sephacryl S200 50/100 (Pharmacia) and separated (buffer: 50 mM Tris, 100 mM NaCl, 0.05 % NaN_3, pH 7.3). The protein concentration in the run-off was monitored by measuring the optical density at 280 nm. The fractions of the first protein peak contained oligomeric urease (molecular weight approx. 550 – 600 kDA), followed by a second peak which was attributed to alkylhydroperoxide-reductase (also an oligomer). Overlapping fractions were discarded, pure fractions (check by electrophoresis on polyacrylamide gel) were purified and concentrated in a vacuum (Speed-Vac) or by ultra filtration. Alkylhydroperoxide-reductase fractions

containing even larger amounts of urease were purified again by means of a β-urease affinity column (antibody Hp8m/1H5-G2B4, Connex, on CNBr-activated Sepharose 4B-matrix, Amersham Pharmacia, coupling according to the manufacturer's instructions).

Isolation of Hsp60 (as an oligomer)

It was not possible to obtain a sufficient quantity of the Hsp60 antigen employing said butanol-extraction method. Thus, the bacteria were ultrasonically separated. Fresh bacteria pellet or pellet obtained after extraction with 10 % butanol was thawed, resuspended at a ration of 1:10 in 150 mM PBS, pH 7.5 and ultrasonically treated (4 x 90 s with 90 s pause each) in a Falcon flask on ice-water in a sonicator (Sonifire) at stage 25 – 30 % (stage 7). After centrifugation at 20,000 g for 40 min, the supernatant was applied on a chromatography column filled with Sephacryl S200 50/100 (Pharmacia) and separated (buffer: 50 mM Tris, 100 mM NaCl, 0.05 % NaN_3, pH 7.3). Hsp60 co-eluted with urease, therefore urease-positive (see above) fractions were purified and again purified on a β-urease affinity column (see above).

Example 4: Generation of polyclonal and monoclonal antibodies (pAK; mAK) against *H. pylori*-specific antigens

Polyclonal antisera against antigens purified as above were produced by pab Productions (Hebertshausen) according to standard methods. Monoclonal antibodies were generated according to methods that have been known to the person skilled in the art (Harlow & Lane, 1988; Peters & Baumgarten, 1990).

Immunisation

The purified urease, Hsp60 and alkylhydroperoxide-reductase as well as recombinant α- and β-urease (Austral Biologics) were used as an immunogene. Per immunogene, 4 to 6 mice (BALB/c x C57 Black, first filial generation, 8 to 12 weeks old) were immunised (priming) and boosted 3 to 4 times in intervals of about four weeks. Per injection 200 to 300 µl antigen-solution (25-50 µg antigen

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per animal) were emulsified at a ratio of 1:1 with complete (priming) or incomplete (boost) Freund's adjuvant (Difco) and 100 µl per mouse were injected intraperitoneally. Before fusion, blood was taken retro-orbitally from the mice and the antibody titre was determined from the antiserum obtained therefrom by means of enzyme-linked immunosorbent assay (ELISA, see below).

Fusion and fusion screening

2 to 3 days after the last boost, the spleen cells of the immunised mice were fused with the myeloma cells P3x63Ag8.653 (ATCC CRL-1580; Kearney et al., 1979) at a ratio of 5:1 with polyethylene glycol 4000. The fused cells were suspended with hypoxanthine-aminopterin-thymidine supplement (100x concentrate, Sigma) in cloning medium (RPMI 1640 Medium + 20 % FCS + 200 U IL-6/ml) and cultured (37°C, 6 % CO_2, 95 % relative humidity) with a cell density of 2-6x10^4 cells/dish on 96-dish micro-titre plates.

After about 10 days, the supernatant of the culture was screened in ELISA to detect antibodies with the desired specificity.

Establishing and cultivating the hybridomas

Clones producing antigen-specific antibodies were re-cloned twice in a cloning medium with hypoxanthine-thymidine supplement (100x concentrate, Sigma) according to the principle of limiting dilution (Coller & Coller, 1983). Then, the monoclonal final clone was adapted to the flat-bottle culture in RPMI 1640 medium with 10 % FCS and expanded for the cryoconservation of 5 to 10 aliquots with 2 to 5x10^6 cells each and for the production of culture supernatants containing antibodies.

ELISA

In order to determine the titre and test the culture supernatants for antigen-specific antibodies, direct ELISA was selected. The coating of the ELISA plates (MaxiSorb; Nunc) was carried out over night at 5°C with an antigen suspension

(2-6 μg antigen/ml carbonate buffer, 0.1 M, pH 9.5). In order to block the binding sites that are still free, 200 μl PBS were pipetted with 2 % skimmed milk powder (weight:volume) per dish and incubated at room temperature for 1 hour. The incubation of 100 μl of the culture supernatant was carried out at room temperature for 1 to 2 hours. The bound antibodies were detected by adding a secondary antibody (rabbit-anti-mouse IgG-POD, DAKO) conjugated with horse-radish peroxidase (POD). In the next step, the POD converts the colourless substrate tetramethylbenzidine (TMB, Sigma) into a blue product. After 5 to 10 minutes or as soon as the negative control also has a slight blue-staining, the reaction was stopped by adding 1 N sulphuric acid (100 μl per dish). The intensity of the colour reaction was measured in the ELISA reader (MWG Spektral). Measurement was carried out at 455 nm against the reference wavelength of 620 nm. Between the single steps, the ELISA plate was washed two to three times with 250 μl PBS with 0.025 % Tween 20 (vol.:vol.).

Results

All in all, 24 monoclonal antibodies against β-urease, 6 monoclonal antibodies against α-urease, 8 monoclonal antibodies against the 26kDa-protein and 10 monoclonal antibodies against Hsp60 were generated. From this repertory of antibodies those monoclonal antibodies were selected that had the lowest detection limit for the respective antigens.

Example 5: Purification of monoclonal antibodies (mAK) from hybridoma-culture supernatants and generation of conjugates

The mAK were purified from hybridoma-culture supernatants by means of the protein-G affinity chromatography (modified according to: Pharmacia Biotech, 1994).

The culture supernatants were filtered (0.8 μm) and carried directly over the protein-G matrix. Washing was carried out with Tris/HCl until the signal at the detector had been in the background again. Eluting was conducted with 0.1 M glycine/ HCl, pH 3.0, the detection of the protein concentration was conducted in an eluate through optical density at 280 nm. All fractions in the range of the single

elution signal may be used. The possible contamination with bovine IgG from the addition of serum may be regarded as uncritical since the detection antibodies found do not cross-react with bovine Ig.

The coupling of monoclonal antibodies to biotin and POD was carried out according to methods in the literature (Harlow & Lane, 1988).

Example 6: Characterising the monoclonal antibodies

Isotyping

The murine mAK were isotyped with the isotyping kit IsoStrip by Boehringer Mannheim (Mannheim).

Immunoblotting

For immunoblotting 30 to 50 µg purified antigen or 300 µg *H. pylori*-lysate per gel were cooked in reducing sample buffer (Laemmli, 1970) and applied on a 12 % preparative SDS-polyacrylamide mini-gel (8.6x7, 7x0.1 cm, Biometra). The electrophoretic separation was carried out at 25-30 mA/gel.

Then, the proteins separated in the gel (antigens) were immobilised in the semidry-blot-method on a nitrocellulose membrane.

The membrane was blocked with 2 % skimmed-milk powder in PBS for 30 min at room temperature and then washed three times for 5 min with TBS/Tween 20 (0.2 %). For the following incubation step, the membrane was fixed in the Accutran cross-blot-screening unit (Schleicher and Schüll) using a grid plate with 34 cross channels. 250 µl TBS/Tween 20 were put in the cross channels and 250 µl each of the hybridoma culture supernatants to be tested were added. Incubation was carried out under agitation for 2 hours at room temperature.

After washing the membrane three times (see above), it was incubated for 1 hour with POD-conjugated secondary antibodies (rabbit-anti-mouse IgG-POD, DAKO).

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The membrane was washed three times and the immune complex was visualised by adding the 3,3-diaminobenzidine-substrate solution (DAB,Sigma).

Characterisation of antibody-antigen-interactions by means of epitope mapping

In order to identify the antibody binding-site to a protein, overlapping peptide sequences of *H. pylori* proteins α- and β-urease Hsp60 and 26kDa-protein (shifted against each other with two amino acids each) with 12 amino acid residues were synthesised to a solid phase and coupled covalently with a cellulose acetate membrane (Jerini GmbH, Berlin). These membranes were incubated with hybridoma culture supernatants and the coupled antibodies were then blotted on nitrocellulose membranes in the semidry-blot method. The membrane was blocked and the immobilised antibodies were detected with a POD-labelled secondary antibody (rabbit-anti-mouse IgG-POD, DAKO) as described in 5.2.

Determination of the detection limit for *H. pylori* antigen in ELISA

Between the single steps the ELISA plate was washed 2 to 3 times with 300 µl PBS with 0.025 % Tween (washing buffer) (vol.:vol.) The ELISA plates (MaxiSorb; Nunc) were coated for 1 hour at 37°C with 100 µl of a solution of a polyclonal rabbit-anti-*H. pylori* antigen (pAK; approx. 10 µg antibody/ml carbonate buffer, 0.1 M, pH 9.5). In order to block the binding-sites that are still free 200 µl 150 mM PBS with 2 % skimmed-milk powder (weight:volume) were pipetted per dish and incubated for 30 min at room temperature. 50 ng/ml purified *H. pylori* antigens solved in 150 mM PBS were diluted adding 0.1 % skimmed-milk powder in 1:2 steps. The buffer served as a negative control. Then, 100 µl culture supernatant diluted 1:10 of the mAK against the same antigen, which is to be analysed, was added and incubated for 30 for 60 min at room temperature. The detection of the antibody was carried out as described in 3.4. The lowest concentration in which an extinction was still detected that was larger than or the same as twice the control was accepted as detection limit.

Table 1: Results for characterising mAK

fusion/clone	Ag-Specificity	Isotype	IB (Ag)	IB (HP-lysate)	NWG (ng Ag/ml)
HP8m/4H5-D4-C9	β-urease	IgG1, κ	+/-	+/-	0.6
HP9.1m/2D1-F6-G1		IgG1, κ	-	+/-	0.3
HP9.1m/3C2-F8-E2		IgG1, κ	+/-	+/-	1.2
HP16.1m/3G1-H2-D7		IgG2a, κ	+	+	0.6
HP15m/3E8-D9-D6	alkylhydro-peroxide-reduktase	IgG1, κ	+	+	0.6
HP15m/3F5-D5-B6		IgG1, κ	+	+	0.3
HP16m/2A5-E6-E5	Hsp60	IgG1, κ	+	+	3
HP18.1m/3F11-G11-H11		IgG1,κ	+	+	3
HP18.1m/4D9-B9-A2		IgG1, κ	+	+	3

Abbreviations: Ag antigen; IB Immunoblot; NWG detection limit

Results

Table 1 summarizes the results of the isotyping, the immunoblotting and the determination of the detection limit for the mAK listed in Table 1.

Table 2: Results of the epitope mapping of monoclonal antibodies against *H. pylori* antigens

Ag-specificity	fusion/clone	epitope (AS-position)	AS-sequence
β-urease (large subunit)	HP8m/1H5-G2-B4	negative	
	HP8m/4H5-D4-C9	negative	
	HP9m/2B12-G7-B12	369-375	VGEVITR
α-urease (small subunit)	HP9m/2E7-B8-G10	negative	
	HP9m/1H7-C6-D5	negative	
alkylhydroperoxide-reduktase	HP15m/3E8-D9-D6	negative	
	HP15m/3F5-D5-B6	negative	
	HP15m/4H12-A4-D5	negative	
		143-149	LPLGRNA
	HP15m/1H7-H3-B7		
Hsp60	HP16m/2A5-E6-E5	negative	
	HP16m/1D10-A8	negative	

Abbreviations: Ag antigen; AS amino acid; negative: epitope could not be mapped.

For two anti-α-urease mAK and two anti-Hsp60 mAK it was not possible to map an epitope by immunoblotting. Only one epitope each could be mapped for three mAK directed against β-urease and four mAK directed alkylhydroperoxide-reductase (see Table 2). Thus, this method did not seem to be suitable for predicting possible overlaps of the epitopes of antibodies having the same specificity.

Example 7: Characterisation of antibody-antigen-interactions by means of surface-plasmon-resonance spectroscopy

By measuring the overlaps of the epitopes with SPR-spectroscopy provides information on the simultaneous access of antibody-epitopes. Thus, suitable antibody pairs for the development of ELISA and the quick assay may be found (Fägerstam LG et al., 1990, Malmqvist M., 1996).

Conduction of the surface-plasmon-resonance spectroscopy at Pharmacia BIAcore

All steps were conducted on a Pharmacia Biacore Processing Unit CA 186 according to standard protocols (NHS/EDC, BIAcore Methods Manual). First, a polyclonal rabbit-anti-mouse antibody was immobilised on the dextran matrix of the BIAcore CM5 glass carrier chip. By adding the first *H. pylori* antigen-specific monoclonal antibody (1. MAK; 100 µg/ml; 10 µl), it reacted with the immobilised catching antibody and the respective change was measured at the detector (ΔRU_1; RU Resonance Units). After adding the *H. pylori* antigen (45 µg/ml; 5 µl), it reacted with the first mAK. Then, the remaining free catching antibody was blocked with an unspecific mouse-antibody (1 mg/ml; 10 µl) and oligomeric epitopes by adding another first mAK (100 µg/ml; 10 µl). After the reaction then following of the second *H. pylori*-antigen-specific antibody (2^{nd} mAK; 100 µg/ml; 10 µl) with the bound antigen, the change in the signal of the detector (ΔRU_2) was determined anew and was compared in percentage to the change at the detector after adding the 1^{st} mAK ($\Delta RU_2/\Delta RU_1$). A ratio of $\Delta RU_2/\Delta RU_1 < 10$ % indicated overlapping epitopes. (Epitopes of an antigen are considered to be overlapping when the first antibody has occupied all epitopes that are to be bound by it and obstructs or inhibits the subsequent binding of the second antibody). In addition, it is possible to calculate the values for the velocity constants of the adsorption and desorption of antibodies by means of kinetic measurements.

Results

Twelve monoclonal anti-urease anti bodies and five antibodies against alkylhydroperoxide-reductase were tested. By comparing the 144 and,

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respectively, 25 possible combinations with each other, such combinations were determined which had a simultaneous accessibility of the epitopes and the affinity constants of which seemed to be suitable for the use in stool-ELISA. Table 3 shows a selection of the results obtained by the combinations of various antibodies against urease and compares the kinetic constants as regards the suitability of these antibody combinations in stool-ELISA (see Example 9). The suitability of antibody pairs for ELISA, does not have to be limited to independent epitopes. Furthermore, a combination of antibodies recognising partly overlapping epitopes (such as HP.91m-2D1 and HP8m-4H5) can be explained by the fact that oligomeric proteins such as urease have the same epitope several times. This allows for the binding of a catching-antibody to an epitope in ELISA and at the same time the binding of one or several detection antibodies to one or several equal epitopes of the oligomeric protein.

Table 3: Four relations of mAK-pairs determined by means of surface-plasmon-resonance and their behaviour in ELISA.

catcher	k_{off} / k_{on}	detector	k_{off} / k_{on}	epitope	evaluation in ELISA
HP9.1m -2B11	$5.9*10^{-4}/7.3*10^{4}$	HP9.1m -2D1	$1.1*10^{-4}/3.7*10^{4}$	independent	-
HP9.1m -2B11	$5.9*10^{-4}/7.3*10^{4}$	HP9.1m -3G1	$4.9*10^{-4}/7.6*10^{4}$	partly overlapping	-
HP9.1m -2D1	$1.1*10^{-4}/3.7*10^{4}$	HP8m -4H5	$4.5*10^{-6}/4.8*10^{4}$	partly overlapping	+++
HP9.1m -XG1	$3.0*10^{-4}/1.5*10^{5}$	HP9.1m -3G1	$4.9*10^{-4}/7.6*10^{4}$	independent	++
HP8m -4H5	$4.5*10^{-6}/4.8*10^{4}$	HP9.1m -3C2	$2.6*10^{-4}/7.4*10^{4}$	independent	+++

Example 8: Selection of antibody pairs for the use in ELISA with human stool

By means of surface-plasmon-resonance, overlapping epitopes were determined of antibodies which had the lowest detection limits when measured from the culture supernatant. The combinations that had been promising during the measurements (as few overlapping epitopes as possible, high velocity constant for adsorption, low velocity constant for desorption) were tested for their detection limits in ELISA stool.

Example 9: Detection of *H. pylori* in human stool by means of ELISA

Abbreviations: detector: detection-antibody; catcher: catching-antibody

As test samples stool samples of patients of the Klinikum Großhadern, Munich, were used whose infection status with *H. pylori* (category 0 and 4) was determined by means of serologic and histological analyses. Category 0 consisted of patients whose clinical result of serology and histology was clearly negative, while the clinical result of serology and histology of category 4 patients was clearly positive.

Between the single steps, the ELISA plates were washed 2 to 3 times with 250 µl PBS adding 0.025 % (washing buffer 1) or 0.2 % Tween 20 (washing buffer 2; vol.:vol.). The ELISA plates (MaxiSorb; Nunc) were coated for 1 hour at 37°C in 100 µl of an mAK-solution (2.5 µg antibody/ml carbonate buffer, 0.1 M, pH 9.5). In order to block the binding sites that are still free, 200 µl 150 mM PBS were pipetted with 0.2 % fish gelatin or 1 % skimmed-milk powder (weight:vol.) per dish and incubated for 30 min at room temperature. Washing was carried out with washing buffer 1. Human stool was suspended with 150 mM PBS at a ratio of 1:10 (weight:vol.) adding 2 % skimmed-milk powder, purified *H. pylori* antigens solved in 150 mM PBS were added in known concentrations. 100 µl of the suspension per dish were incubated for 1 hour. First, the plate was washed by hand, then it was washed 4 times with a washing buffer. Subsequently, 100 µl of a solution consisting of mAK (0.5 µg antibody/ml PBS) against the same antigen coupled with biotin were added and incubated for 30 to 60 min at room temperature. The detection of the bound antibodies was carried out by adding a

conjugate of streptavidin with POD (DAKO). The POD converts the colourless substrate TMB (Sigma) into a blue product. After 5 to 10 minutes or as soon as the negative control also had a slight blue staining, the reaction was stopped by adding 1 N sulphuric acid (100 µl/dish). The intensity of the colour reaction was measured in the ELISA-reader (MWG Spektral). Measurement was carried out at 455 nm against the reference wavelength of 620 nm.

Table 4: Detection of *H. pylori* antigens in the stool of patients who are clearly negative (category 0) or clearly positive (category 4) by means of ELISA using monoclonal antibodies:

Table 4a: category-0-samples: 0 is false-positive, 1 is right-negative

	[µg Ag/ g stool]			resulting truth-value			
sample	urease	26kDa	Hsp60	urease	26kDa	Hsp60	3-combination
cut-off				1	15	5	
0047	0.6	2.3		1	1		1
0048	0.6	2.3		1	1		1
0051	2.1	22		0	0		0
0057	0.6	2.3		1	1		1
0069	0.6	2.3		1	1		1
0074	0.6	2.3		1	1		1
0087	0.6	2.3		1	1		1
0099	0.6	2.3		1	1		1
0185	0.6	2.3		1	1		1
0186	0.6	2.3		1	1		1
0189	0.6	2.3		1	1		1
0265	0.6	2.3		1	1		1
0298	0.6	2.3		1	1		1
0305	0.6	2.3		1	1		1
0373	0.6	2.3		1	1		1
0074	0.6	2.3	2.3	1	1	1	1
0089	0.6	2.3	2.3	1	1	1	1
0090	0.6	2.3	2.3	1	1	1	1
0091	0.76	2.3	2.3	1	1	1	1
0097	1.6	2.3	2.3	0	1	1	0
0103	1	2.4	15	1	1	0	0
0107	1	12	2.3	1	1	1	1
0114	4	2.3	44	0	1	0	0
0126	0.6	12	2.3	1	1	1	1
0130	0.76	67	2.3	1	0	1	0

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sample	urease	26kDa	Hsp60	urease	26kDa	Hsp60	3-combination
0141	20	220	105	0	0	0	0
0142	1.8	2.3	2.3	0	1	1	0
0144	0.6	2.3	2.3	1	1	1	1
0146	0.6	2.3	2.3	1	1	1	1
0148	0.6	2.3	2.3	1	1	1	1
0168	0.6	2.3	2.3	1	1	1	1
0193	0.6	2.3	20	1	1	0	0
0207	0.6	2.3	20	1	1	0	0
0220	0.6	2.3	2.2	1	1	1	1
0225	0.6	2.3	2.2	1	1	1	1
0231	0.6	2.3	2.2	1	1	1	1
0235	0.6	2.3	2.2	1	1	1	1
0251	0.6	2.3		1	1		1
0258	0.6	95		1	0		0
0274	0.6	2.3		1	1		1

Table 4b: category 4 samples: 0 is false-negative, 1 is right-positive

sample	[µg Ag/ g stool]			resulting truth-value			
	urease	26kDa	Hsp60	urease	26kDa	Hsp60	3-combination
cut-off				1	15	5	
0006	1.9	25	34	1	1	1	1
0010	5.6	91.5	170	1	1	1	1
22	7.8	45	25	1	1	1	1
0350	15.6	198	95	1	1	1	1
0288	18	34	65.9	1	1	1	1
0332	29	360	650	1	1	1	1
0218	7	24	3	1	1	0	1
0285	7.4	115	3	1	1	0	1
0004	9.5	2.3	350	1	0	1	1
11	21	15	26	1	0	1	1
8	105	2.3	89	1	0	1	1
0003	1.5	2.3	10	1	0	1	1
15	2.3	2.3	15	1	0	1	1
3005	1.2	2.3	2.3	1	0	0	1
0002	1.8	2.3	2.3	1	0	0	1
0206	9.6	2.9	3	1	0	0	1
0135	11		3	1	0	0	1
0175	16	2.9	3	1	0	0	1
0005	25	2.3	2.3	1	0	0	1
0278	25.7	4	3	1	0	0	1
0001	127	2.3	2.3	1	0	0	1
0004	127	2.9	3	1	0	0	1
0037	127	2.9	3	1	0	0	1
0294	127	14	2.3	1	0	0	1

0108	17.5		3	1		0	1
28	0.6	69	330	0	1	1	1
0012	0.6	23	8.6	0	1	1	1
0013	0.6	27.7	3	0	1	0	1
6c	0.6	2.8	35	0	0	1	1
18c	0.6	2.3	15	0	0	1	1
53	0.6	2.3	17	0	0	1	1
0164	0.6	2.9	5.9	0	0	1	1
3007	0.6	2.3	2.3	0	0	0	0
9c	0.6	2.3	2.3	0	0	0	0
13c	0.6	2.3	2.3	0	0	0	0
47	0.6	2.3	2.3	0	0	0	0
17	0.6	2.3	2.3	0	0	0	0
18	0.6	2.3	2.3	0	0	0	0
0331	0.6	2.3	3	0	0	0	0
0063	0.6	2.9	3	0	0	0	0
0179	0.6	2.9	3	0	0	0	0
0213	0.6	2.9	3	0	0	0	0
0233	0.6	2.9	3	0	0	0	0
0081	0.6	2.9	14.4	0		1	1

Table 4c: truth-values for the detection of urease, 26kDa-protein and Hsp60

	truth-value			
	urease	26kDa	Hsp60	3-combination
threshold value	1	15	5	
specificity	88 %	90 %	77 %	75 %
sensitivity	57 %	26 %	41 %	75 %
right-negative	35	36	17	30
overall negative	40	40	22	40
right-positive	25	11	18	33
overall positive	44	42	44	44

Abbreviations: 26kDA is the 26kDa-protein; 3-combination is the combination of all three antigens (urease, 26kDa-protein and Hsp60).

In the case of urease, HP8m/4H5 + HP16m/XG1 were used as catching-antibodies and detection-antibodies. The detection limit was at 0.075 ng/ml. In the case of the 26kDa-protein, HP15m/4H12 was used as catching-antibody and HP15m/3E8 as detection-antibody. The detection limit was at 1.5 ng/ml. In the

case of Hsp60, HP18.1m/3F11 was used as catching-antibody and HP16m/2A5 + HP18.1m/4D9 as detection-antibody. The detection limit was at 6 ng/ml.

Results

Tables 4a – 4c show the results of an analysis of stool samples for urease, alkylhydroperoxide-reductase and Hsp60. The third column (3-combination) shows the respective value for the resulting truth-value of the combination of all three antigens (urease, 26Kda-protein, Hsp60).

All in all, 30 out of 40 analysed category-0 control samples (Table 4a) were found to be negative (specificity 75 %), 33 out of 44 category-4 samples (Table 4B) were found to be positive (sensitivity 75 %). In 6 out of 44 samples it was possible to detect all three antigens, in 25 out of 44 samples it was possible to detect urease, in 11 our of 42 samples alkylhydroperoxide-reductase and in 18 out of 44 samples Hsp60 could be detected. As regards the combination of all three antigens (urease, 26kDa, Hsp60), a specificity of 75 % and a sensitivity of 75 % was achieved (Table 4c).

Table 5 shows the result of the analysis of 3 samples taken at three different positions of a patient's stool. The figures correspond to the extinction measured at 650 nm divided by the extinction of a zero-sample, i.e. a multiplication of the background (H). There was an inhomogeneous distribution of the detected antigens.

Table 5: distribution of *H. pylori*-specific antigens in stool samples taken at different positions of the infected patient's stool.

detected antigen	β-urease	26kDa-protein	Hsp60
sample 1	8.6 x H	2.5 x H	1 x H
sample 2	11 x H	1 x H	1 x H
sample 3	3.5 x H	1 x H	1 x H

Example 10: Cloning and sequencing of the functionally variable regions of immunoglobalins of hybridoma cell lines

According to Chomczynski (Chomczynski, 1987), total-RNA was isolated from hybridoma cell lines producing antibodies.

Then, the corresponding cDNA was synthesised according to standard methods (Sambrook et al., 1989).

The DNA regions encoding the kappa light chain as well as the heavy chain-Fd-segment (VH + CH1) of the respective antibodies were amplified by means of PCR. Use was made of the oligonucleotide primer set indicated in Table 6. The cDNA isolated from the hybridoma cell lines was used as a matrix.

The primer set used led to a 5'-*Xho*I and a 3'-*Spe*I cleavage site each in the heavy chain-Fd-fragments as well as to a 5'-*Sac*I and a 3'-*Xba*I cleavage site each in the kappa light chains. 11 different 5'-VH-primers (MVH1-8 and MULH1-3) were combined with the 3'-VH-primer MIgG1 for the PCR amplification of the DNA fragments encoding the heavy chain-Fd. For the amplification of the DNA fragments encoding for the kappa light chains, 11 different 5'-VK-primers (MUVK1-7 and MULK1-4) were combined with the 3'-VK-primer 3'MUCK.

The following temperature programme was used for all PCR amplifications: initial denaturation at 94°C for 3 min, denaturation at 94 °C for 25 s, primer-attachment at 52°C for 60 s, polymerisation at 72°C for 90 s. This programme was maintained for 40 cycles, followed by the subsequent completion of the fragments at 72°C for 10 min.

The results of the PCR amplifications were separated by means of agarose gel electrophoresis and the DNA bands of the expected molecular weight were isolated. Then, a restriction digest was carried out with the isolated bands using the enzymes *Xho*I and *Spe*I (heavy chains) and *Sac*I and *Xba*I (light chains), the fragments obtained were cloned into the plasmid vector Bluescript KS (Stratagene) after cleaving this vector with the restriction enzymes *Xho*I and *Spe*I or *Sac*I and *Xba*I.

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Then, plasmid preparations of the cloned heavy and light chain fragments were sequenced. For every hybridoma cell line sequences were chosen which encode for functionally variable regions of the immunoglobulin heavy and light chain (VH and VL respectively). In this way, it was possible to identify exactly one functional VH-and one functional VL-region for each hybridoma cell line. The functional VH-and VL-sequences are shown in Figures 1-8. Cloning and sequencing was conducted according to standard procedures (Sambrook et al., 1989).

Table 6: list of the functionally variable regions of heavy and light immunoglobulin-chains used for the PCR amplification.

MVH1	(GC)AG GTG CAG CTC GAG GAG TCA GGA CCT
MVH2	GAG GTC CAG CTC GAG CAG TCT GGA CCT
MVH3	CAG GTC CAA CTC GAG CAG CCT GGG GCT
MVH4	GAG GTT CAG CTC GAG CAG TCT GGG GCA
MVH5	GA(AG) GTG AAG CTC GAG GAG TCT GGA GGA
MVH6	GAG GTG AAG CTT CTC GAG TCT GGA GGT
MVH7	GAA GTG AAG CTC GAG GAG TCT GGG GGA
MVH8	GAG GTT CAG CTC GAG CAG TCT GGA GCT
MULK1	GGG GAG CTC CAC CAT GGA GAC AGA CAC ACT CCT GCT AT
MULK2	GGG GAG CTC CAC CAT GGA TTT TCA AGT GCA GAT TTT CAG
MULK3	GGG GAG CTC CAC CAT GGA GWC ACA KWC TCA GGT CTT TRT A
MULK4	GGG GAG CTC CAC CAT GKC CCC WRC TCA GYT YCT KGT
MIgG1	TAT GCA ACT AGT ACA ACC ACA ATC CCT GGG
MUVK1	CCA GTT CCG AGC TCG TTG TGA CTC AGG AAT CT
MUVK2	CCA GTT CCG AGC TCG TGT TGA CGC AGC CGC CC
MUVK3	CCA GTT CCG AGC TCG TGC TCA CCC AGT CTC CA
MUVK4	CCA GTT CCG AGC TCC AGA TGA CCC AGT CTC CA
MUVK5	CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA
MUVK6	CCA GAT GTG AGC TCG TCA TGA CCC AGT CTC CA
MUVK7	CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA
MULH1	GGG CTC GAG CAC CAT GGR ATG SAG CTG KGT MAT SCT CTT
MULH2	GGG CTC GAG CAC CAT GRA CTT CGG GYT GAG CTK GGT TTT
MULH3	GGG CTC GAG CAC CAT GGC TGT CTT GGG GCT GCT CTT CT
3`MUCK	GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A

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